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Application of Statistics in 2x2 Crossover Bioequivalence Studies

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Abstract: *Bioequivalence studies are intended to assess the pharmaceutical equivalence of test product with innovator product. Well-designed bioequivalence study explores the bioavailability and adverse events occurred during study and guide safety measures through healthy human subjects participated in the study. To achieve these clinical objectives, statistical methods are useful and important to conclude them as well. An adequate sample size enrollment is important while designing a bioequivalence study. By applying statistical methods to pharmacokinetic parameters in a randomized study helps to conclude bioequivalence of test to reference (innovator) products. This paper provides the information on appropriate statistical application in a randomized 2x2 crossover average bioequivalence study.*

Keywords: *Sample Size, Randomized, ANOVA, Confidence Interval, Bioequivalence*

1. INTRODUCTION

Bioequivalence is a term in pharmacokinetics used to assess the expected in vivo biological equivalence of two proprietary preparations of a drug. If two products are said to be bioequivalent it means that they would be expected to be, for all intents and purposes, the same. Pharmacokinetic is an important study in a multi-phase clinical trial research conducted for evaluation of new drug (NDA) in human subjects and in a generic drug (ANDA) development as well.

The United States Food and Drug Administration (FDA) has defined bioequivalence as, "the absence of a significant difference in the rate and extent to which the active ingredient or active moiety in pharmaceutical equivalents or pharmaceutical alternatives becomes available at the site of drug action when administered at the same molar dose under similar conditions in an appropriately designed study."[1]

A pilot bioequivalence study is usually conducted on 12 or 18 human subject to estimate the within (σ_{WT}^2, σ_{WR}^2) and between (σ_{BT}^2, σ_{BR}^2) subject drug variability and to obtain log-transformed averages of test (μ_T) and reference product (μ_R). A bioequivalence study design includes number of periods, sequences, treatments, washout periods, treatment conditions (fasting or after food), fluid intake with dosage, time and type of food and fluids throughout the study day. A well planned bioequivalence study consist the adequate number of pre and post-dose blood samples to compensate for between-subject differences in absorption and elimination rate and thus enable accurate determination of the maximum concentration of the Active Pharmaceutical Ingredient (API) in the blood (C_{max}) and terminal elimination rate constant (K_{ele}) in all subjects. The adequacy of blood sample collection depends on the nature of the API and the input function from the administered dosage form. A sampling period extending to at least four to five elimination half-lives of the drug is usually sufficient. The results of sampling times are known as 'drug concentration measurements' and processed to estimate pharmacokinetic parameters.

Overall purpose of a bioequivalence study is to compare the log-transformed pharmacokinetic bioavailability measure (e.g., AUC and Cmax) after administration of the test and reference (innovator) products. The bioequivalence comparisons normally rely on (1) a criterion, (2) a confidence interval for the criterion, and (3) a predetermined BE limit.[2]

The paper is structured in the following way. Description of a single dose crossover bioequivalence study is given in Section 2, including some fundamental concepts regarding sample size estimation and randomization. In Section 3, the proposed statistical model and methodology for performance

measurement is provided. In section 4 the results obtained from predication model are summarized. Conclusions are given in Section 5.

2. BIOEQUIVALENCE STUDY DEFINITIONS

In this section, some basic definitions of single dose crossover bioequivalence study were revised from Schuirmann, D.J. (1987), Schuirmann, D.J. (1989), Hauck, W.W., and S. Anderson (1992), Chinchilli, V.M., and J.D. Esinhart (1996), Chen, M.-L., R. Patnaik, W.W. Hauck, D.J. Schuirmann, T. Hyslop, R.L. Williams, and the FDA Population and Individual Bioequivalence Working Group (2000) and Center for Drug Evaluation and Research (CDER), USFDA (2001) and (2003). The definitions and notations presented in this section were used throughout this work and are essential to understand the proposed model.

2.1. Average Bioequivalence

There are three types of bioequivalence evaluation individual, population and average. Average bioequivalence is widely used in the pharmaceutical industry.

2.2. Sample Size

Sample size plays vital role in bioequivalence study. There are several methods available for sample size estimation. Intra/inter subject variability, point estimate of test to reference product, power, alpha value, confidence bound are the essential parameter for sample size calculation. This parameter information can be obtained from literature, previous pilot study, in some instances when actual data information is not available, use of reasonable assumptions are also in practice. An adequate sample size helps to find out true bioequivalence of test product.

Additive equivalence test for mean difference with normal data is useful for sample size estimation of a 2x2 crossover bioequivalence study. The hypotheses for the equivalence test are:

$$H_0: \mu_{diff} < \theta_{LowerCI} \quad \text{or} \quad \mu_{diff} < \theta_{upperCI}$$

$$H_1: \theta_{LowerCI} \leq \mu_{diff} \leq \theta_{upperCI}$$

A minimum 24 human subjects are essential to enroll in a standard 2x2 crossover study.

2.3. Randomization

Need of randomized bioequivalence or clinical trial is to have unbiased experimental control. Randomization provides for unbiased estimates of error variance and for independence of errors. It avoids predictability treatment assignment to subjects.

For a 2 period, 2 sequence crossover bioequivalence study randomization schedule must be balanced.

Randomization for standard 2x2 crossover bioequivalence study design			
Sequence	**Period 1**	**Washout Period**	**Period 2**
Sequence 1 (AB or TR) (n subjects)	Test Product Data: Y_{i11}	**> 5 half-life of drug**	Reference / Innovator Product Data: Y_{i21}
Sequence 2 (BA or RT) (n subjects)	Reference / Innovator Product Data: Y_{i12}		Test Product Data: Y_{i22}

2.4. Pharmacokinetic Parameters

Pharmacokinetics, sometimes abbreviated as **PK** is a branch of pharmacology dedicated to determining the fate of substances administered externally to a living organism. Pharmacokinetics provides a mathematical basis to assess the time course of drugs and their effects in the body. It enables the following processes to be quantified [3]:

Absorption

Distribution

Metabolism

Excretion

The basic pharmacokinetic parameters in bioequivalence study are as follows:

Primary variables: $C_{max,}$ AUC_{0-t} and $AUC_{0-\infty}$

Secondary variables: $T_{max,}$ $AUC_{\_\%Extrap}$, $t_{1/2}$ and K_{el}

C_{max} : Maximum observed plasma drug concentration over a specified time period.

T_{max} : Observed time to reach maximum drug concentration (C_{max}).

AUC_{0-t} : Area under the plasma concentration-time curve measured to the last quantifiable concentration, using the trapezoidal rule.

$AUC_{0-\infty}$: AUC_{0-t} plus additional area extrapolated to infinity, calculated using the formula $AUC_{0-t}+C_t/K_{el}$, where C_t is the last measurable drug concentration and Kel is the elimination rate constant.

K_{el} : Apparent first-order terminal elimination rate constant calculated from a semi-log plot of the plasma concentration versus time curve, using the method of least square regression.

$t_{1/2}$: Elimination half-life as determined by quotient Ln(2)/ K_{el}

$AUC_{\_\%Extrap}$: Percentage of Area Under the Plasma Concentration extrapolated from AUC_{0-t} to $AUC_{0-\infty}$.

2.5. Analysis of Variance (ANOVA)

ANOVA is perhaps the most powerful statistical tool also widely used in clinical trial data analysis. For 2x2 crossover design, the unpaired two-sample *t* statistic is equivalent to a special case of analysis of variance. The concept of the analysis of variance is to study the variability in the observed data partitioning the total sum of squares (SS) of the observation into components of the fixed effects and the random errors.[4]

2.6. Interval Hypothesis and Bioequivalence Criteria

Schuirmann (1981, 1987) first introduced the two-one sided procedure for assessing average bioequivalence between formulations. The proposed two-one sided procedure suggest the conclusion of equivalence of μ_T and μ_R at α level of significance. if and only if, below hypothesis is rejected at predetermined α level of significance:

H_{01}: $\mu_T - \mu_R \leq \theta_{Lower}$ and H_{02}: $\mu_T - \mu_R \geq \theta_{Upper}$

Versus *Versus*

H_{a1}: $\mu_T - \mu_R > \theta_{Lower}$, H_{a2}: $\mu_T - \mu_R < \theta_{Upper}$.

Based on above hypothesis bioequivalence can be concluded as,

If a confidence interval of 100(1-2α)% for the difference ($\mu_T - \mu_R$) or for the ratio (μ_T / μ_R) is within acceptable limits as recommended by the regulatory agency, i.e., within intervals [θ_{inf}: θ_{sup}] or [δ_{inf}: δ_{sup}], respectively, then the conclusion is that there is bioequivalence; otherwise, the conclusion is for the non-existence of bioequivalence.[5]

3. THE PROPOSED MODEL

The assessment of average bioequivalence is based on the comparison of bioavailability parameters (i.e. AUC and C_{max}) between formulations. It is known that no two formulations will have exactly the same bioavailability profiles. Therefore, if the profiles of the two formulations differ by less than a (clinically) meaningful limit, the profiles of the two formulations may be considered equivalent.[4] This concept Schuirmann (1981) first introduced the use of interval hypotheses for assessing average bioequivalence. It is equally important to establish a true equivalence between formulations without losing efficacy of the drug, which is intended for treatment in real life. The proposed model ensures to evaluate bioequivalence with an adequate 2x2 crossover experimental design.

3.1. Methodology

The objective of this paper is to develop an experimental design that allows decision makers to measure equivalence between formulations. Here we use ANOVA and interval hypotheses approach. We applied methodology to experimental data for evaluation of our objective.

3.1.1 Analysis of Variance (ANOVA)

The analysis of variance (ANOVA), equivalent to two one-sided tests, is performed on ln-transformed pharmacokinetic parameters C_{max}, AUC_{0-t} and $AUC_{0-\infty}$ for drug. The ANOVA model includes sequence, subject nested within the sequence, period and formulation as factors. Sequence effect tested using subject nested within sequence as the error term, at 10% level of significance. The

remaining three factors tested against the error variance obtained from the ANOVA at 5% level of significance. Each ANOVA includes calculation of least square means (LSM), the difference between formulation LSM, and the standard error (SE) associated with these differences. (Refer Table 1, Table 2 and Table 3).

3.1.2 Confidence Interval

90% confidence intervals are constructed for the least square mean differences (Test- Reference) of the ln-transformed pharmacokinetic parameters C_{max}, AUC_{0-t} and $AUC_{0-\infty}$.The confidence limits are expressed as a percentage of the LSM of the Reference product. The exponential or the antilogs of these limits are used to construct the 90% confidence intervals for the ratio of geometric least square means of the test and reference products. (Refer Table 4).

3.1.3 Bioequivalence Acceptance Criteria

The ratio of geometric least squares means for the ln-transformed parameters of drug must be within 80.00 to 125.00 % Bioequivalence range and corresponding 90% confidence interval calculated from the exponential of the difference between the test and reference product for the ln-transformed parameters C_{max}, AUC_{0-t} and $AUC_{0-\infty}$ of drug must be within the 80.00 to 125.00 % Bioequivalence range. (Refer Table 4).

3.2. Formulation of Problem

To assess bioavailability and bioequivalence of a new formulation test drug 'A' compared with innovator drug 'B' in healthy, adult, human subjects.

A single center, randomized, single dose, open-label, analyst-blind, two-treatment, two-period, two-sequence, crossover, comparative bioavailability and bioequivalence study design was used to assess the objective.

4. STATISTICAL ANALYSIS OF PHARMACOKINETIC PARAMETERS

Log-transformed pharmacokinetic parameters C_{max}, AUC_{0-t} and $AUC_{0-\infty}$ were used as dependent variable in an ANOVA model. The ANOVA model includes sequence, subject nested within the sequence, period and formulation as factors. The statistical analysis performed using General Linear Model procedure (Proc GLM) in SAS software.

Table1. *ANOVA for Ln-transformed C_{max} (The GLM Procedure)*

Class Level Information		
Class	**Levels**	**Values**
Form	2	A B
Period	2	1 2
Seq	2	AB BA
Subject	14	1 2 3 4 5 6 7 8 9 10 11 12 13 14

Number of Observations Read	28
Number of Observations Used	28

Source	**DF**	**Sum of Squares**	**Mean Square**	**F Value**	**Pr > F**
Model	15	8.95757213	0.59717148	13.72	<.0001
Error	12	0.52226057	0.04352171		
Corrected Total	27	9.47983270			

R-Square	**Coeff Var**	**Root MSE**	**LnCmax Mean**
0.944908	3.238300	0.208619	6.442225

Source	**DF**	**Type III SS**	**Mean Square**	**F Value**	**Pr > F**
Seq	1	0.87878668	0.87878668	20.19	0.0007
Subject(Seq)	12	8.05277538	0.67106462	15.42	<.0001
Period	1	0.00005280	0.00005280	0.00	0.9728
Form	1	0.02595727	0.02595727	0.60	0.4549

Tests of Hypotheses Using the Type III MS for Subject(Seq) as an Error Term					
Source	**DF**	**Type III SS**	**Mean Square**	**F Value**	**Pr > F**
Seq	1	0.87878668	0.87878668	1.31	0.2748

	LnCmax	**Standard**	**H0:LSMEAN=0**	**H0:LSMean1=LSMean2**
Form	**LSMEAN**	**Error**	**Pr > |t|**	**Pr > |t|**
A	6.47267201	0.05575566	<.0001	0.4549
B	6.41177717	0.05575566	<.0001	

Period	LnCmax LSMEAN	Standard Error	H0:LSMEAN=0 Pr > |t|	H0:LSMean1=LSMean2 Pr > |t|
1	6.44085132	0.05575566	<.0001	0.9728
2	6.44359786	0.05575566	<.0001	

Parameter	Estimate	Standard Error	t Value	Pr > |t|
Test-Ref	0.06089484	0.07885041	0.77	0.4549

Table2. *ANOVA for Ln-transformed AUC_{0-t} (The GLM Procedure)*

Class Level Information		
Class	Levels	Values
Form	2	A B
Period	2	1 2
Seq	2	AB BA
Subject	14	1 2 3 4 5 6 7 8 9 10 11 12 13 14

Number of Observations Read	28
Number of Observations Used	28

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	15	7.43105958	0.49540397	26.36	<.0001
Error	12	0.22548930	0.01879078		
Corrected Total	27	7.65654888			

R-Square	Coeff Var	Root MSE	LnAUCt Mean
0.970549	1.562002	0.137079	8.775883

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Seq	1	0.72917905	0.72917905	38.81	<.0001
Subject(Seq)	12	6.64160087	0.55346674	29.45	<.0001
Period	1	0.01362305	0.01362305	0.72	0.4112
Form	1	0.04665661	0.04665661	2.48	0.1411

Tests of Hypotheses Using the Type III MS for Subject(Seq) as an Error Term					
Source	DF	Type III SS	Mean Square	F Value	Pr > F
Seq	1	0.72917905	0.72917905	1.32	0.2734

Form	LnAUCt LSMEAN	Standard Error	H0:LSMEAN=0 Pr > |t|	H0:LSMean1=LSMean2 Pr > |t|
A	8.81670359	0.03663602	<.0001	0.1411
B	8.73506273	0.03663602	<.0001	

Period	LnAUCt LSMEAN	Standard Error	H0:LSMEAN=0 Pr > |t|	H0:LSMean1=LSMean2 Pr > |t|
1	8.79794075	0.03663602	<.0001	0.4112
2	8.75382557	0.03663602	<.0001	

Parameter	Estimate	Standard Error	t Value	Pr > |t|
Test-Ref	0.08164086	0.05181116	1.58	0.1411

Table3. *ANOVA for Ln-transformed AUC_{0-inf} (The GLM Procedure)*

Class Level Information		
Class	Levels	Values
Form	2	A B
Period	2	1 2
Seq	2	AB BA
Subject	14	1 2 3 4 5 6 7 8 9 10 11 12 13 14

Number of Observations Read	28
Number of Observations Used	28

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	15	7.48375356	0.49891690	25.94	<.0001
Error	12	0.23081674	0.01923473		
Corrected Total	27	7.71457030			

R-Square	Coeff Var	Root MSE	LnAUCinf Mean
0.970080	1.570601	0.138689	8.830334

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Seq	1	0.48792600	0.48792600	25.37	0.0003
Subject(Seq)	12	6.94117726	0.57843144	30.07	<.0001
Period	1	0.02919496	0.02919496	1.52	0.2415
Form	1	0.02545534	0.02545534	1.32	0.2724

Tests of Hypotheses Using the Type III MS for Subject(Seq) as an Error Term					
Source	**DF**	**Type III SS**	**Mean Square**	**F Value**	**Pr > F**
Seq	1	0.48792600	0.48792600	0.84	0.3765

Form	**LnAUCinf LSMEAN**	**Standard Error**	**H0:LSMEAN=0 Pr > |t|**	**H0:LSMean1=LSMean2 Pr > |t|**
A	8.86048542	0.03706628	<.0001	0.2724
B	8.80018221	0.03706628	<.0001	

Period	**LnAUCinf LSMEAN**	**Standard Error**	**H0:LSMEAN=0 Pr > |t|**	**H0:LSMean1=LSMean2 Pr > |t|**
1	8.86262432	0.03706628	<.0001	0.2415
2	8.79804330	0.03706628	<.0001	

Parameter	**Estimate**	**Standard Error**	**t Value**	**Pr > |t|**
Test-Ref	0.06030321	0.05241964	1.15	0.2724

Table4. *Schuirmann`s Two One Sided t-tests and Classical 90% Confidence Intervals For Ln-transformed Data (Acceptance Criterion: 80.00%-125.00%)*

PK Parameter	**LSM Test**	**LSM Reference**	**LSM Diff**	**n1**	**n2**	**DF**	**GeoMean Test**	**GeoMean Ref**
LnCmax	6.4727	6.4118	0.0609	7	7	12	647.2108	608.9750
LnAUCt	8.8167	8.7351	0.0816	7	7	12	6745.9904	6217.1242
LnAUCinf	8.8605	8.8002	0.0603	7	7	12	7047.9031	6635.4529

PK Parameter	**Ratio (%)**	**90% C.I. Lower Limit**	**90% C.I. Upper Limit**	**MSE**	**Intra-CV%**	**Power (%)**	**Bioequivalence**
LnCmax	106.28	92.34	122.31	0.0435	21.09	73.64	Yes
LnAUCt	108.51	98.94	119.00	0.0188	13.77	97.26	Yes
LnAUCinf	106.22	96.74	116.62	0.0192	13.94	97.01	Yes

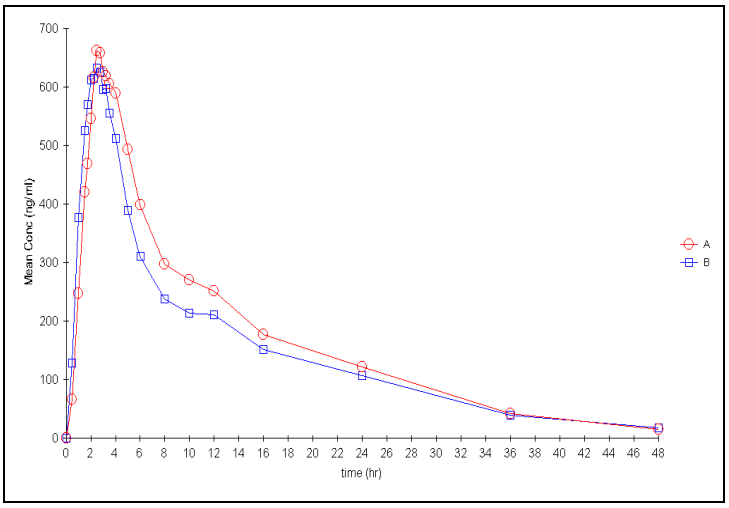

Fig1. *Comparative Linear Plot of Time versus Mean Concentration of drugs*

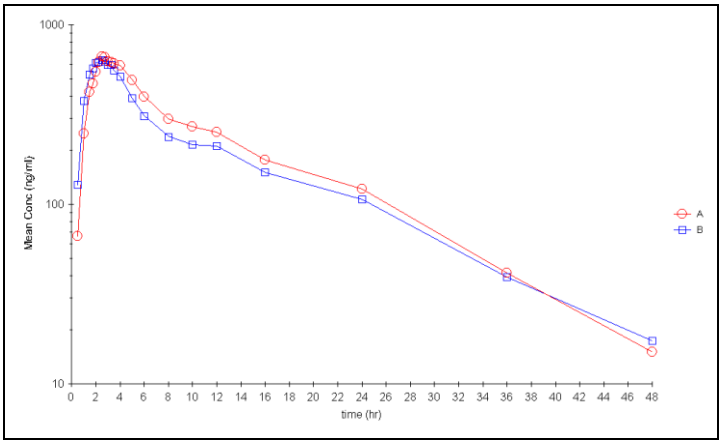

Fig2. *Comparative Log-Linear Plot of Time versus Mean Concentration of Drug*

5. DISCUSSION & CONCLUSION

The paper established a 2x2 crossover model that allows decision makers to evaluate drug bioavailability and bioequivalence. It was predefined to compare the log-transformed pharmacokinetic bioavailability measure AUC and Cmax after administration of the test product 'A' and reference (innovator) product 'B' in healthy human subjects. Drug development and its appropriate evaluation are crucial to know the drug efficacy for the noble cause of improving living of human being. ANOVA technique separated the total variability in a set of data into component parts represented by statistical model.

A 2 period, 2 sequence and 2 treatment crossover bioequivalence study on 14 subjects shows that effects in the ANOVA model (sequence, period, formulation) are statistically non-significant ($p>0.05$). The test to reference ratio for $\ln C_{max}$ is 106.28% and its associated 90% confidence interval is 92.34% to 122.31%. The test to reference ratio for $\ln AUC_{0\text{-}t}$ is 108.51% and its associated 90% confidence interval is 98.94% to 119.00%. The test to reference ratio for $\ln AUC_{0\text{-}t}$ is 106.22% and its associated 90% confidence interval is 96.74% to 116.62%. The intra-subject variability for the pharmacokinetic parameter $C_{max,}$ $AUC_{0\text{-}t}$ and $AUC_{0\text{-}inf}$ is 21.09%, 13.77% and 13.94% respectively. The power for pharmacokinetic parameter $C_{max,}$ $AUC_{0\text{-}t}$ and $AUC_{0\text{-}inf}$ is 73.64%, 97.26% and 97.01% respectively.

The 90% confidence interval for test to reference ratio are within the bioequivalence acceptance criteria of 80.00 to 125.00% for each of the log transformed pharmacokinetic parameter C_{max}, $AUC_{0\text{-}t}$ and $AUC_{0\text{-}inf}$. Hence it is concluded that the test product 'A' is bioequivalent to the reference (innovator) product 'B'.

Statistical methods applied in this bioequivalence study facilitate to conclude a true equivalence between the two formulations. The Decision Maker (D.M) should use this efficient model in bioequivalence evaluation of two drug formulations.

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